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Systemic Activation of the IFN System by Exposure of Natural Epithelia to Physiologic (Low Dose) Levels of IFN

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Splenocytes treated with very low concentrations of cross-reactive recombinant human interferon alpha A/D (IFN A/D) provided significant protection in mice against the effects of Semliki Forest virus (SFV), ($p<0.001$). In addition, groups of mice inoculated intranasally with splenocytes treated with 30 IU/ml of IFN showed greater protection against encephalitis and death from SFV than did those inoculated with 100 IU/ml of IFN-treated splenocytes, ($p<0.025$). Thus the response was biphasic. In 13 experiments mice treated with low concentrations of IFN A/D or mouse IFN-α/β in their drinking water were protected from SFV ($p<0.0005$). The responses occurred over a narrow concentration range and were biphasic. Thus higher concentrations of IFN were not protective, ($p<0.4$). Duplication of the biphasic effects by recombinant human IFN strongly suggests they were activated by the IFN. No IFN was detected in

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the serum of mice even when drinking water contained 10^4 IU/ml. In contrast, 1 ml of similarly low concentrations of IFN-α/β (10^1 to 10^3 IU/ml) inoculated twice daily ip at the site of infection, beginning two days before infection and ending two days after, did not protect. In fact, 5,000 to 50,000 units twice daily were required to obtain the same level of protection. These findings indicate that the protection likely was not due to the direct effect of IFN having diffused into the circulation and then transported to the site of infection or target organs. Thus, these results strongly suggest the IFN protects against systemic infection from SFV by activating a potent, but not yet characterized novel defense system. This system originates in the mucosa and likely involves a type of cellular communication and amplification. Knowledge of this system could play a role in developing and maintaining natural resistance to infections.

ANNUAL REPORT ON CONTRACT NOOO14-89-J-1962

INTRODUCTION

Many hormone and lymphokine-like substances naturally exert highly active, dose-dependent, biphasic effects (1). We found previously that syngeneic, non-cytotoxic lymphocytes treated with low (10-50 IU/ml) concentrations of IFN inoculated ip could protect mice from a lethal SFV infection (2). Treatment with high (>50 IU/ml) concentrations, on the other hand, caused a diminished effect. These findings indicate for the first time the biphasic nature of a novel IFN-activated protective response that can operate in vivo. Based on these and other in vitro studies (3), we hypothesized that lymphocytes treated with low concentrations of IFN interacted with the peritoneal epithelium and transferred amplified antiviral resistance to the mouse. This raised the possibility that epithelial surfaces of the upper alimentary and/or respiratory mucosa exposed to lymphocytes treated with low concentrations of IFN or with IFN itself could initiate antiviral resistance in a similar biphasic fashion. To test these possibilities, we gave mice splenocytes treated with varying concentrations of IFN intranasally or IFN alone in varying concentrations in drinking water and measured their resistance to SFV infection.

MATERIALS AND METHODS

Interferons

The human affinity purified recombinant IFN A/D, specific activity ($6x10^7$ U/mg protein) was kindly supplied by Dr. Sidney Pestka (Department of Molecular Genetics and Microbiology, UMDNJ, Robert Wood Johnson School of Medicine, Piscataway, NJ).

Natural mouse IFN-a/b (specific activity= 7.7x106 IU/mg protein) was kindly supplied by Dr. S. Baron (Department of Microbiology, UTMB, Galveston, Texas). Briefly, this IFN was prepared by infecting mouse L-929 cells, in the absence of any serum, with Newcastle disease virus. After subjecting the supernatant fluids harvested 24 hr post-infection to pH 2 for 5 days, the antiviral titer in a VSV plaque reduction assay was $6x10^5$ IU/ml.

Virus Preparations and Use

The Semliki Forest virus preparations used as challenge in these experiments were prepared from brains harvested from moribund ICR mice. The stock preparations titered approximately 5 x 10^8 pfu/ml on BHK cells. Two LD50s, or a $10^{-6.5}$ to $10^{-7.5}$ dilution of the stock, were used for ip challenge in most experiments. In general, older mice, e.g., six-week-old mice, were challenged with the $10^{-6.5}$ dilution, and younger mice, e.g., 4-week-old mice, were challenged with a $10^{-7.5}$ dilution of the stock. If too much virus was injected for a mouse of a given age, and 100% mortality resulted in all groups by 4 to 6 days post infection, no IFN concentrations were protective. If too little virus was given, it was impossible to get meaningful statistics with the size of the groups used, because of small differences in mortality rates. Reproducibility consistently occurred when 70 to 90% of the control mice died by 8 to 9 days post infection.

Interferon Concentrations in Drinking Water

IFN concentrations in drinking water were determined by a standard VSV plaque reduction assay at least twice in each experiment by assaying the water directly when concentrations were 10 IU/ml or above and when necessary at lower concentrations, by assaying the individual 10X IFN stocks used to make them. The latter was done because of problems encountered with toxicity of water. Additional assays showed that the concentrations of IFN in water samples that could be assayed did not change during the time the mice were drinking the water. We assume the same was true for the lower concentration. The medium used for cell culture, IFN and virus preparation, and dilutions was Earle's minimal essential medium (EMEM) supplemented with 2% fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 ug/ml).

Animals

Four- to six-week old, Harlan Sprague-Dawley female mice (Houston, Texas) were randomized into groups, 8 to 13 per group depending on the experiment, and treated ad libitum with the indicated concentrations of IFN in their drinking water. Supernatant fluid from L-929 cell cultures was used as mock IFN (0 units concentration). The IFNs were stable in water, which contained at least 0.01% bovine serum albumin or 0.2% FBS, for at least 5 days. Polyurethane bottles and plastic stoppers containing freshly prepared IFN from a 10X IFN stock maintained at 4°C were changed daily with sterile bottles and stoppers. Individual mice drank approximately 5 ml of water

per 24 hours up until the time those that were going to die became sick, or about 4 to 6 days post-infection. Since treatment could be reduced to 2 days post-infection without changing the results, all mice in a group consumed approximately the same total quantities of IFN during the critical period of treatment; for example, mice receiving 30 ml took in approximately 150 IU/24 hr, or 750 units total. The experiments were ended 10 days post-infection, since early studies showed mice surviving 10 days continued to survive.

RESULTS AND DISCUSSION

Specifically, ICR mice were treated with pooled, recombinant human IFN A/D-treated splenocytes (2 x 10^6/treatment) intranasally before and after a lethal SFV infection ($2LD_{50}$/mouse) (4,5). Biphasic responses occurred and were similar to that observed when the cells were given ip (2). Analysis of the significance of differences and trends among the mortality proportions (dead/total number of mice in each treatment group) by chi^2 and Fisher's exact test, as warranted, indicated a difference among all of the proportions ($p=0.004$). Mice receiving splenocytes treated with 30 IU/ml of IFN A/D were significantly protected ($p<0.001$) from the lethal SFV infection when compared to control mice given mock-treated splenocytes (Fig. 1). Those mice receiving splenocytes treated with higher concentrations of IFN A/D, however, were not protected ($p<0.025$), demonstrating again the biphasic nature of this response. Importantly, the results suggest the protective effect activated was most likely systemic because of the distal application of the splenocytes relative to the site of challenge.

For the same purpose, we also studied whether mouse IFN-a/b or recombinant human IFN A/D present in low concentrations in drinking water (4,5) of mice could result in similar biphasic protection. Figure 2A-D shows the results of 4 (representative of 13) experiments in which ICR mice were given varying concentrations of mouse IFN-a/b (Fig. 2A and B) or cross-reactive human IFN A/D (Fig. 2C and D) in drinking water before and after ip infection with SFV (2 LD_{50}). These experiments indicated the biphasic effects were most likely due to IFN, since recombinant IFN worked as well as natural mouse IFN, and also that the ranges of effective IFN concentrations were narrow, consisting approximately of 10-100 IU/ml. In addition, the data shown in Figure 2B are representative of two experiments in which four groups of mice received no measurable amounts of IFN based on direct titration of the water or fresh 10x stock that was added to

the water. Since there were no significant variations seen between these groups and numerous other mock-treated control groups, the possibility that the biphasic effect was due to random variation in survival rates in mice receiving the same challenge of SFV is unlikely.

Also, since there were no significant differences due to length of IFN treatment (treatments were systematically reduced from five days prior through six days post-challenge to two days prior through two days post-challenge), or to the type of IFN used, the results of all 13 experiments were graphed (Fig. 3). We used analysis of variance and the Kruskal-Wallis non-parametric test for statistical significance of a biphasic response initiated by the low concentrations of IFN. Table 1 gives the P values for the protective effects of low levels (1-300 IU/ml) vs. the controls, which were 0.0005 and 0.0004, respectively. In addition, the P values of these analyses for the low concentration effect versus the high concentration effect were 0.04 and 0.03, respectively. Thus, these results indicate that the protection observed again is biphasic. The variability between the range of concentrations of IFN in Figures 2 and 3 most likely reflects variations encountered in titrations of the IFN concentrations present in the water or fresh 10x stock used in each experiment, which is routinely plus or minus 0.5 $\log_{10}$ in our assay. Because of this variation and the narrow ranges of the modulatory effect (Fig. 3), we found that a range of IFN concentrations that included the 0.5 $\log_{10}$ error had to be used to consistently reproduce the biphasic effect in this system.

The overall effectiveness of low concentration IFN treatment is shown in Figure 4, which compares control mortality rates and optimum protection rates in each of the experiments. The result of examining the groups in this fashion was an overall control survival rate of 16%, while the survival rate at the optimum low concentration of IFN- treated mice was 70%. Thus, the mechanism activated by optimum levels of low concentrations of IFN obviously is potent. In contrast, repeated studies revealed that the same low and higher concentrations of IFN injected once daily (1.0 ml) ip in the same time intervals relative to infection by SFV, as reported above, only protected at high concentrations (5×10^3 IU/ml to 5×10^4 IU/ml). This result would be expected from past experience in the prophylactic use of IFNs.

Our correlated results therefore show that exposure of mucosal surfaces to 1) splenocytes treated with low concentrations of IFN, or 2) low concentrations of IFN directly can systemically activate a potent protection mechanism in mice. This response was biphasic in nature and resembles other tightly controlled physiological responses, such as those of hormones and lymphokines (1). In this regard,

the molar concentrations of the IFNs that were active in this system interestingly are roughly equivalent to serum levels of peptide hormones, or to approximately 10^{-11} mol/L (6). Since the same effects were observed with natural or recombinant IFN, IFN itself seems likely to be responsible for initiating the effects. Because of the very low concentrations of IFN required to activate this system, whether it was activated by splenocytes treated with IFN before inoculation and washed exhaustively or with IFN applied directly, a considerable amount of amplification must have occurred to mediate the systemic protective effect. In support of this, we observed that IFN inoculated ip at low concentrations for the same time period as in the experiments above, did not demonstrate the same biphasic effect. In fact, to achieve the same protective results, 10,000 to 100,000 units of IFN per day were required. This amount of IFN inoculated ip per day would result in a serum concentration of approximately 100 to 1000 IU/ml (7). Thus, the site of application and concentration of IFN applied or naturally present at various times appears important in the activation and amplification of this system.

One explanation for the observed protection could be the development of a hyper-responsive IFN-producing state or priming caused by the direct effect of very low concentrations of IFN. Thus, cells that had been exposed to low concentrations of IFN and infected by virus would respond by producing sufficiently higher levels of IFN that could account for the protection. This explanation is unlikely since: 1) in the serum of mice no IFN was detectable that could diffuse to the site of infection even following administration of 10,000 IU/ml in drinking water (ca. 50,000 IU/day); 2) there were no differences in the serum levels of IFN found 18 hours after infection in control mice or in those treated with any of the concentrations of IFN; and further, 3) if treatment was stopped the day of infection by which time priming should be in effect (8), no protection from mortality was observed (9). Even if some of the observed protection were due to hyper-responsiveness, it would most likely result from an indirect effect of the IFN initiated at a site distal to infection. To further support a causal argument for the indirect effect of IFN, we have found that as few as 1.8 units of IFN/day given intranasally at a concentration of 30 IU/ml (0.2 ml/applied/3x/day starting at 2 days prior to infection and continuing 2 days after) resulted in the same biphasic protective effect (10). It would be difficult to explain how this small quantity or even higher quantities of IFN (50-500 units/day) given by the oral or nasal route could result in the observed protection by the direct effects of the IFN.

Another possible explanation for the amplified protection could be that an IFN-induced signaling mechanism was activated by the locally applied IFN. Numerous published studies support this interpretation, beginning with the observation that IFN-

treated cells could communicate antiviral activity to neighboring cells in the absence of the direct effect of IFN (11). Amplification has been shown to occur within this system by demonstrating that the kinetics of development of an antiviral state in a mixed human cell culture was controlled by the fastest responding cells (12). Additionally, as few as 10% of the cells controlled the magnititude of the antiviral response in a population treated with IFN (13). More recently, we have shown that this likely communicative activity can occur in vivo, and importantly the observed protection was biphasic and accomplished with syngeneic, noncytotoxic lymphocytes treated with low concentrations (10-50 IU/ml) of IFN and washed exhaustively (2). Based on these observations, we are presently investigating whether the amplified systemic signaling process involves circulating leukocytes and/or humoral factors, especially hormones and lymphokines.

Since the conditions for activation of this biphasic system exist naturally due to the presence of ubiquitous IFN inducers (i.e., normal microbial flora), or very early during virus infection, this type of exposure may be responsible for a significant proportion of what is called natural resistance. Specifically, IFN present in high levels at the earliest sites of infection (14) would decrease in concentration as radial diffusion occurred. Thus, a large number of surrounding cells would be exposed to a gradient of IFN concentrations, one low range of which would result in enhanced protection by activating the biphasic system. If only low levels of IFN were induced in the proper range, the system would also be activated. We do not know why higher levels of IFN were not effective; however, some type of feedback mechanism or receptor modulation may be involved. This interpretation can help account for the inability of the host to cope with large (high dose) challenges of viruses or other organisms known to induce IFNs, since locally high concentrations of IFN would develop rapidly.

Overall, these studies indicate that a novel, IFN-activated mechanism initiated by organisms infecting or interacting with cells of the mucosa, possibly including lymphatic tissue, such as tonsils and adenoids, likely plays a major role in the development of the body's internal defense against these and other subsequent types of infecting organisms. The potential efficacy of using low concentrations of IFN for therapeutic treatment of feline leukemia virus and human immunodeficiency virus infections in humans has been reported (15). Oral administration of IFN to neonatal mice in higher concentrations prior to infection with vesicular stomatis virus, also administered orally, has also been shown to protect (16).

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17. Care and use of all experimental animals were performed in accordance with The University of Texas Medical Branch's institutional guide lines.

Figure Legends

Fig. 1. Protection of mice from SFV infection by splenocytes treated with low concentrations of IFN. In two experiments, groups of eight female, five-week-old ICR mice were inoculated intranasally three times daily (0.2 ml) with $2x10^6$ splenocytes freshly harvested, ficol-hypaque-enriched, and treated for 4 hr with mock or recombinant human IFN-alpha A/D at the indicated concentrations. Chi^2 analysis of the mortality difference between groups receiving mock and 30 IU/ml IFN treated splenocytes resulted in a P value of <0.001. Similar analysis of the groups receiving splenocytes treated with 30 IU/ml or 100 IU/ml resulted in a P value of <0.025.

Fig. 2. Four representative experiments out of 13 showing protection of mice from SFV by low concentrations of IFN present in drinking water (9). Mice in panels a and b received natural mouse IFN a/b (6) while those in panels c and d received recombinant human IFN A/D (4). The number of mice per group in a=8, b=10, c=12, d=13. In these experiments, all IFNs were given ad libitum in drinking water for 6 days prior and 6 days post-infection.

Fig. 3. The results of 13 experiments showing protection of mice by low, but not high, concentrations of IFNs present in drinking water. The 13 experiments were lumped together because no significant differences were observed in the overall biphasic protection between groups receiving natural mouse IFN-a/b or recombinant human IFN-alpha A/D, nor were their differences resulting from the times of treatment which ranged from 6 days pre-infection through 6 days post-infectin to two days pre-infection through 2 days post-infection. The data are analyzed in Table 1.

Fig. 4. Comparison of the survival rates of groups of mice treated with optimal low concentrations of IFN in the 13 experiments.

Table 1. Statistical analysis of the effects of low concentrations of IFN

IFN (IU/ml) concentration	% Mortality and S.E.	No. of groups	Total mice
0	84.13 ± 3.17	21	246
1-300	56.75 ± 4.27	40	411
1000	81.25 ± 6.25	4	40

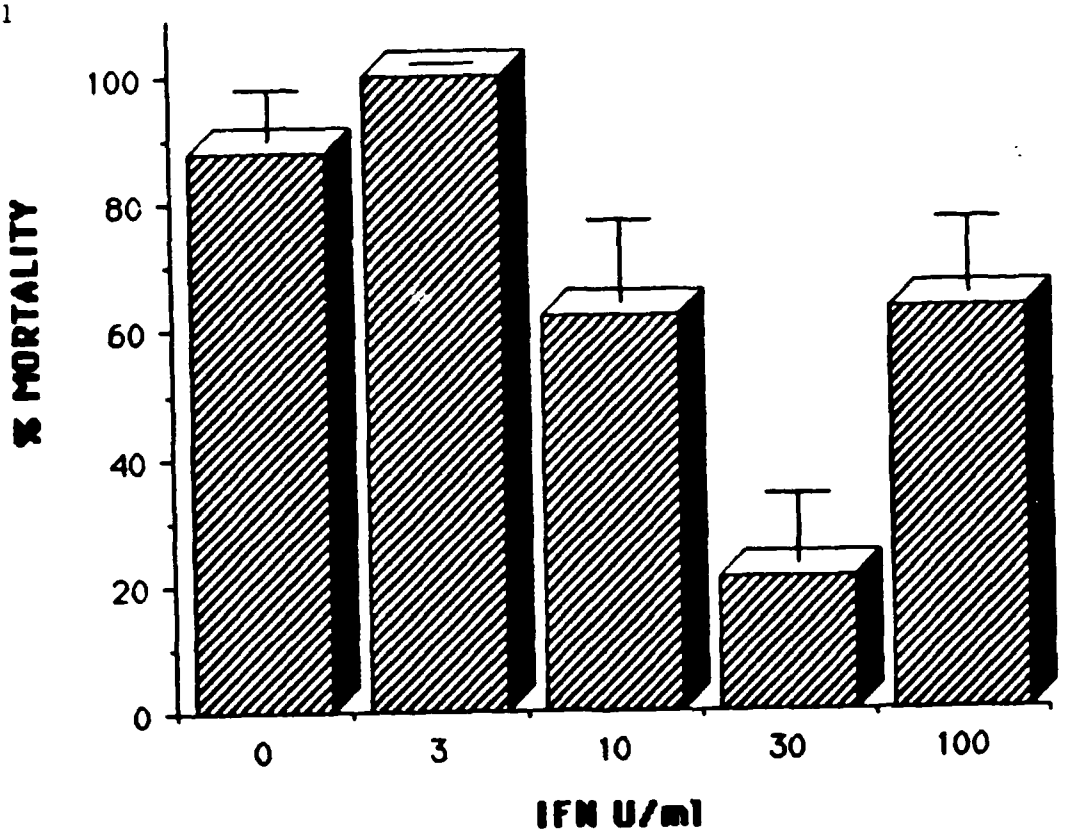
P (0 vs. 1-300 IU/ml) <0.0005 by ANOVA test.

P (0 vs. 1-300 IU/ml) <0.0002 by Kruski Wallis non-parametric

P (1-300 vs. 1000 IU/ml) = 0.03 by ANOVA test.

P (1-300 vs. 1000 IU/ml) = 0.04 by Kruski Wallis test.

Figure 1



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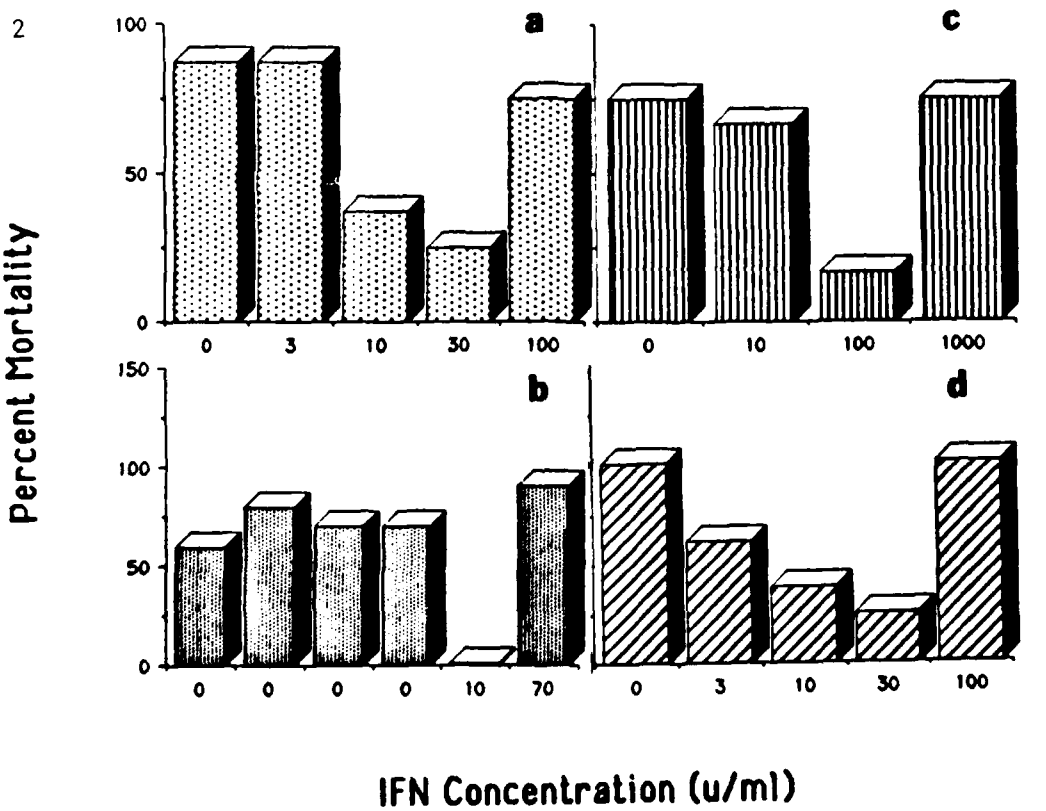

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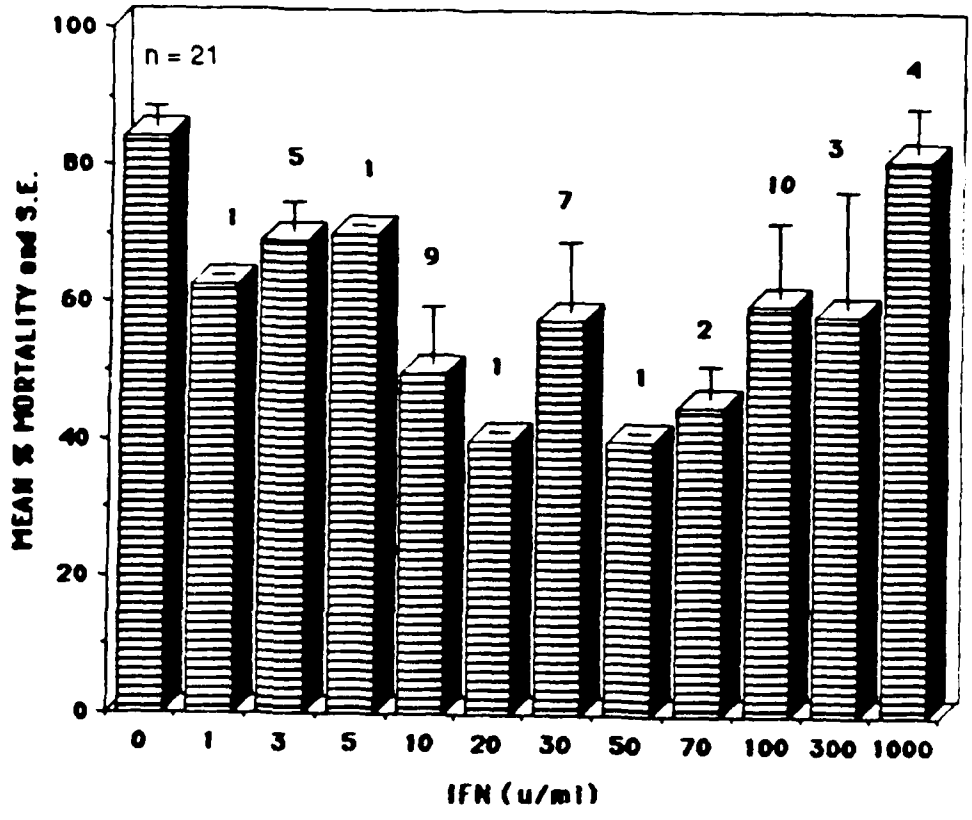

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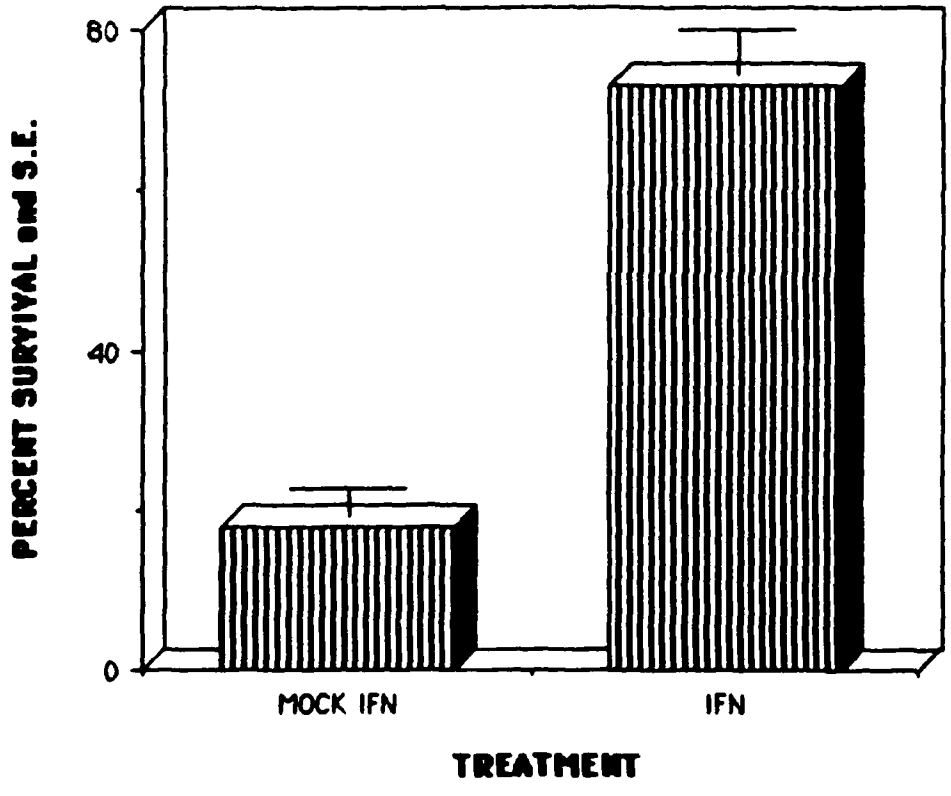

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